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A scalable method for O-antigen purification applied to various *Salmonella* serovars

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Abstract

The surface lipopolysaccharide of gram-negative bacteria is both a virulence factor and a B cell antigen. Antibodies against O-antigen of lipopolysaccharide may confer protection against infection, and O-antigen conjugates have been designed against multiple pathogens. Here, we describe a simplified methodology for extraction and purification of the O-antigen core portion of *Salmonella* lipopolysaccharide, suitable for large-scale production. Lipopolysaccharide extraction and delipidation are performed by acetic acid hydrolysis of whole bacterial culture and can take place directly in a bioreactor, without previous isolation and inactivation of bacteria. Further O-antigen core purification consists of rapid filtration and precipitation steps, without using enzymes or hazardous chemicals. The process was successfully applied to various *Salmonella enterica* serovars (Paratyphi A, Typhimurium, and Enteritidis), obtaining good yields of high-quality material, suitable for conjugate vaccine preparations.

Keywords

O-antigen; Lipopolysaccharide; *Salmonella*

Lipopolysaccharide (LPS) is the major component of the surface of most Gram-negative bacteria and is recognized by immune cells as a pathogen-associated molecule. LPS consists of a polysaccharide chain of repeating units, the O-antigen, linked to a core oligosaccharide containing 10–12 sugar units. The core is covalently bound through 2-keto-3-deoxyoctonate (KDO) to lipid A. Lipid A is highly conserved and exerts endotoxic activity, while the O-antigen chain differs between serovars and is a major contributor to the serological specificity of bacteria. Antibodies against the O-antigen may confer protection against infections [1] and vaccines have been developed by conjugation of O-antigen to carrier proteins to enhance immunogenicity. O-antigen-based conjugate vaccines have been proposed for many human pathogens including *Salmonellae* [2–4], *Shigella* species [5–7], and *Escherichia coli* [8]. A critical precursor for the production of conjugate O-antigen vaccines is the purification of O-antigen separated from all impurities including traces of LPS, as LPS contamination of the vaccine would lead to undesirable local and systemic reactogenicity.

In most published protocols for producing O-antigen-based vaccines, O-antigen purification is carried out after LPS extraction and hydrolysis. Various procedures have been proposed for LPS isolation [9,10], including methods using trichloroacetic acid [11], ether [12], EDTA [13], aqueous butanol [14], Triton X-100/Mg²⁺ [15], cold ethanol [16], diethylene glycol or aqueous pyridine [17], extraction in water at 100 °C [18], and methanol–chloroform [19]. Other extraction methods, employing phenol, chloroform, petroleum–ether [20], and methanol [21], have been described specifically for rough LPS, while a further method using sodium dodecyl sulfate [22] has been developed to efficiently extract both smooth and rough LPS. However, because of its higher yield, the most frequently employed method for LPS extraction is the Westphal procedure [23], commonly known as hot phenol extraction. Some modifications have been introduced into the basic protocol to reduce contamination and produce higher yields [24–26] and the hot phenol extraction method, despite its cancerous and toxic nature, is commercially used to extract large amounts of LPS.

With the aim of purifying O-antigen for the production of conjugate vaccines, hot phenol extraction has been applied to *Salmonella* Typhimurium [2], *Salmonella* Paratyphi A [3], *Salmonella* Enteritidis [4], *Shigella* [5], *Bordetella* [27], and *E. coli* [8]. After the initial step of LPS extraction, hydrolysis is performed with acetic acid [2,3,5,8,23] or anhydrous hydrazine [3,28] usually followed by treatments with enzymes and size-exclusion chromatography to remove contaminants such as proteins and nucleic acids. The traditional process for O-antigen isolation and purification overall is complex and time consuming and involves manipulation of large volumes of hazardous phenol and toxic intermediate LPS. For this reason, there is a clear need for safer improved processes of O-antigen extraction and purification from Gram-negative bacteria, especially in relation to large-scale production.

In 1988 Kondo and Hisatsune proposed a procedure for obtaining O-antigen from *Vibrio cholerae* whole cells without first extracting the LPS [29]. Bacterial cells were harvested, homogenized in hot water, and hydrolyzed with 5% acetic acid at 120 °C. The new methodology was successfully applied to various Gram-negative bacteria [30], verifying that the sugar composition was identical to the O-antigen obtained with the hot phenol technique.

Here, we describe a further improvement of the direct extraction method, which employs acetic acid hydrolysis at 100 °C taking place directly in the bacterial culture. For industrial sterilize-in-place fermenters, the hydrolysis can be performed directly in the fermentation vessel, minimizing handling and operator exposure to pathogenic bacteria. This treatment cleaves the labile linkage between KDO, at the proximal end of the core oligosaccharide, and lipid A, releasing the O-antigen chain attached to the core sugars (Fig. 1). The hydrolyzed O-antigen chain and core sugars are indicated here as OAg for simplicity. The process was successfully applied to OAg from *S. Paratyphi* A (O:2), *S. Typhimurium* (O:4,5), and *S. Enteritidis* (O:9). The O-antigen chains from these three *Salmonella enterica* serovars share a common backbone, but differ in their α-(3,6)-dideoxyhexose-(1,3)-linked to D-mannose branching moieties. Dideoxyhexoses such as paratose, abequose, or tyvelose confer *Salmonella* serogroup specificity (O:2, O:4,5, and O:9, respectively; Fig. 1) [3,31,32].

We also describe a new process for *Salmonella* OAg purification, using simple precipitation and filtration steps suitable for large-scale preparations. The combination of both extraction and purification procedures yielded good quantity and quality of OAg with all tested *Salmonella* strains.

Materials and methods

Chemicals

Acetic acid (AcOH), ammonium hydroxide (NH₄OH) solution, sodium hydroxide (NaOH) solution, sodium phosphate dibasic (Na₂HPO₄), calcium chloride dihydrate (CaCl₂·2H₂O), citric acid monohydrate, phenol, semicarbazide hydrochloride, sodium acetate (AcONa), and 2-keto-3-deoxyoctonate ammonium salt (KDO) were from Sigma; sodium chloride (NaCl) and sulfuric acid (H₂SO₄) were from Merck; absolute ethanol and sodium phosphate monobasic monohydrate (NaH₂PO₄·H₂O) were from Carlo Erba; acetonitrile (CH₃CN) was from LC-MS Chromasolv. NaOH solution (J. T. Baker) and sodium acetate salt (preweighed reagent; Dionex) were used for high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis. D-Glucose monohydrate, D-galactose, N-acetyl-D-glucosamine (Sigma), and L-rhamnose monohydrate and D-mannose (Fluka) were used as monomer standards for the same analysis.

Origin of bacterial strains

The attenuated strain *S. Paratyphi* A CVD1901 was obtained from Dr. Myron M. Levine (Center for Vaccine Development, University of Maryland, College Park, MD, USA) [33]. The clinical isolates *S. Typhimurium* D23580 and *S. Enteritidis* D24359 were obtained from the Malawi–Liverpool–Wellcome Trust Clinical Research Programme (Blantyre, Malawi). D23580 is a typical and representative Malawian invasive NTS isolate belonging to the ST313 sequence type [34,35]. *S. Typhimurium* 2189 and 2192 are animal isolates [36,37] and were obtained from the University of Calgary (Calgary, AB, Canada). The laboratory strains *S. Typhimurium* NVGH1792 and LT2 [38] and *S. Enteritidis* CMCC4314 (or ATCC4931) were obtained from the Novartis Master Culture Collection. The animal isolates *S. Enteritidis* SA386 and SA710 were obtained from Quotient Bioresearch (UK).

Purification materials

These materials were used for the purification of OAg samples: Sartocoon Slice 200, 200-cm² filter area, 30-kDa Hydrosart membrane (308 144 59 02 E–SW; Sartorius) was used for purification steps by tangential flow filtration (TFF). Sartobind S MA75 (Sartorius) and 0.2-μm PES syringe filter (Nalgene; 194-2520) were used for the filtration steps.

Growth conditions and fermentation

All bacterial strains were grown and fermented in chemically defined media [39] containing 13.3 g/L KH₂PO₄, 4 g/L (NH₄)₂HPO₄, 1.7 g/L citric acid monohydrate, 2 mM MgSO₄·7H₂O, 1 mL/L trace elements, 1 mL/L trace vitamins, and 3% glycerol. Trace elements solution contained 2.5 g/L CoCl₂·6H₂O, 15 g/L MnCl₂·4H₂O, 1.5 g/L CuCl₂·H₂O, 3 g/L H₃BO₃, 2.5 g/L Na₂MoO₄·2H₂O, 2.5 g/L Zn(CH₃-COO)₂·H₂O, and 100 mM ferric citrate. Trace vitamins solution contained 50 g/L thiamine, 10 g/L nicotinic acid, 10 g/L Capanthotenate, 10 g/L pyridoxine-HCl, and 1 g/L cyanocobalamin. Glycerol was used as the carbon source with additional medium supplementation depending upon the strain:

- *S. Paratyphi* A CVD 1901, 0.04% guanine, 0.13 g/L asparagine, and 0.13 g/L methionine (for growth in a 7-L bioreactor); 0.08% guanine, 0.5 g/L asparagine, and 0.5 g/L methionine (for growth in a 75-L bioreactor);
- *S. Typhimurium* and *S. Enteritidis*, no additional supplementation.

Bacteria were grown in a 7-L bioreactor (EZ-Control; Applikon) to OD of approximately 35 as previously described [39]. *S. Paratyphi* A CVD1901 was also grown at 30-L scale in a 75-L bioreactor (Biostat D-DCU D75; Sartorius). Growth conditions were controlled, keeping

the temperature at 37 ± 0.5 °C, air flow rate at 1 vvm, pH at 6.7 (by addition of NH_4OH 28%), and $p\text{O}_2$ saturation at 30% (by agitation and pressure controls in cascade).

Direct OAg extraction

AcOH was added to the fermentation culture at a final concentration of 1–2% (v/v), with a resulting pH in the range of 3.5–4.7, and incubated at 100 °C for the optimal time of hydrolysis determined for each strain. At the end of hydrolysis, addition of 28% NH_4OH increased the pH to around 6 and the supernatant was collected by centrifugation. This solution is indicated in the text as “post hydrolysis supernatant”. The hydrolysis was performed at 2-mL scale in 4-mL Wheaton glass vials, using either a boiling water bath or an oven; at 2-L scale using a heat-jacketed glass round-bottom flask connected to a condenser; and at 30-L scale directly in the bioreactor, maintaining the temperature constant at 100 ± 0.5 °C through the double jacket and the closed loop thermostat system.

OAg purification

In a typical purification (Table 1), 1 L of “post hydrolysis supernatant” was concentrated 5- to 10-fold (depending on the concentration of OAg and/or impurities) using TFF with a 30-kDa molecular weight cutoff (MWCO), 200- cm^2 Hydrosart membrane, followed by diafiltration with 20 diavolumes of 1 M NaCl and another 10 diavolumes of water. During diafiltration, retentate volume was kept constant, maintaining input pressure (P_{in}) of 1.8–2.0 bar and transmembrane pressure (TMP) of 1.1–1.2 bar. Retentate, indicated here as “TFF-1 retentate”, was then collected.

Citrate buffer (200 mM, pH 2.7) was added with continuous stirring to the “TFF-1 retentate” to give a final citrate concentration of 20 mM and a final pH of the OAg solution around 3. After mixing at room temperature for 30 ± 5 min, the supernatant was collected by centrifugation (12,000g at 15 °C for 30 min) removing a substantial precipitate containing contaminating proteins and nucleic acids. The solution containing OAg, designated as “post-pH 3”, was further purified by cation-exchange chromatography, through a Sartobind S MA75 filter. This step was needed only when the residual protein content after the precipitation at pH 3 was >3% (w/w respect to total sugar content). Before proceeding with filtration, the Sartobind S cartridge was equilibrated with 20 mM citrate, pH 3. The OAg sample was then loaded and collected in the flow through. Proteins were eluted with 20 mM citrate, 1 M NaCl, pH 3. The resulting OAg solution was referred to as “post-Sartobind S”.

The following solutions were then added to the “post-Sartobind S” (or “post-pH 3”) while stirring at room temperature: 500 mM Na_2HPO_4 , absolute ethanol (EtOH), and 5 M CaCl_2 to give 18 mM Na_2HPO_4 , 24% EtOH, (v/v), and 200 mM CaCl_2 concentrations in the final mixture. During 30 ± 5 min of mixing, a precipitate containing nucleic acids formed and the OAg was recovered in the supernatant (“post-EtOH/ CaCl_2 ”) after centrifugation (12,000g at 15 °C for 30 min).

TFF at 30 kDa (Hydrosart membrane area of 200 cm^2) was then performed. The solution was concentrated up to 10 times and then diafiltered against 10 diavolumes of water, keeping retentate volume constant ($P_{\text{in}} = 1.8\text{--}2.0$ bar and $\text{TMP} = 1.1\text{--}1.2$ bar). Finally, the purified OAg solution (“TFF-2 retentate”) was filtered through a 0.22- μm filter (PES Nalgene; “post-0.22 μm ”) and stored at 4 °C.

Analytical methods

The phenol sulfuric acid assay [40] was used for quantifying total sugar content. Protein concentration was measured by micro-BCA, using bovine serum albumin as a reference following the manufacturer’s instructions (Thermo Scientific). Analysis of nucleic acids was

performed by UV spectroscopy at a wavelength of 260 nm assuming that a nucleic acid concentration of 50 µg/mL gives an OD₂₆₀ of 1. Protein and nucleic acid contents were expressed as ratio percentage relative to sugar content (w/w). Chromogenic kinetic LAL (*Limulus* amoebocyte lysate) was used to measure endotoxin level (Charles River Endosafe-PTS instrument).

“Post hydrolysis supernatant” and “post-EtOH/CaCl₂” samples were analyzed after desalting against water on a HiTrap desalting column, 5 mL, prepacked with Sephadex G-25 Superfine (GEHealthcare), to avoid interference from the matrix in the colorimetric methods.

¹H nuclear magnetic resonance (NMR) spectroscopy

NMR analysis on the liquid state was performed to confirm the identity of the OAg samples by detecting typical signals of the OAg chain, confirming the presence of the characteristic sugars, as well as for calculating the molar ratio of paratose (Par), tyvelose (Tyv), or abequose (Abe) to rhamnose (Rha), by comparing the integrals of the two peaks corresponding to Par (Tyv or Abe)-H6 and Rha-H6.

Dried OAg samples (2.5 mg total sugar) were solubilized in 650 µL deuterated water (D₂O; Aldrich) and transferred to 5-mm NMR tubes. Proton NMR experiments were recorded at 25 °C on a Varian VNMR5-500 spectrometer, equipped with a Pentaprobe. Acquisition time of 5 s, relaxation delay of 15 s, and number of scans of 64 were set for recording the spectra. For data acquisition and processing VNMRJ version 2.2 revision C and Mestrenova 6.1 (Mestrelab Research) were used, respectively. One-dimensional proton NMR spectra were collected using a standard one-pulse experiment. Chemical shifts were referenced to hydrogen deuterium oxide (HDO) at 4.79 ppm (¹H).

Proton high-resolution magic angle spinning (HR-MAS) NMR experiments were collected on entire bacteria in heterogeneous phase to verify if the OAg was still attached on the bacterial membranes. *S. Typhimurium* bacteria of D23580 strain were grown overnight in 5 mL of LB medium up to OD 4–5 (1 OD unit corresponds to about 3 × 10⁹ CFU/mL), harvested, and killed in 3 mL of 10 mM phosphate-buffered saline, pH 7.2, containing 1% formaldehyde (w/w). The suspension was incubated for 1 h at 37 °C. A second preparation of cells was subjected to acid treatment (2% AcOH v/v, 100 °C, 2 h). Both bacterial samples were pelleted by centrifugation (4500g for 2 min) and washed three times with 10 mM potassium phosphate buffer in D₂O. Each compact pellet was transferred into a Kel-F disposable insert for a 50-µL volume and then spun in a 4-mm MAS ZrO₂ rotor (Bruker).

Proton HR-MAS NMR experiments were recorded by a Bruker Avance III 400-MHz spectrometer, using a Bruker 4-mm HR-MAS probe. Samples were spun at 4.5 kHz and recorded at 25 °C. Proton spectra of bacterial cells were acquired with diffusion filter pulse sequence to remove sharp lines arising from low-molecular-mass species in solution. To facilitate the profile comparison, an additional ¹H NMR experiment of purified OAg from D23580 in liquid solution was also recorded at 25 °C by the same spectrometer, using a 5-mm broadband probe (Bruker). Approximately 5 mg of OAg was solubilized in 0.75 mL of D₂O (Aldrich) and the solution inserted into a 5-mm NMR tube (Wilmad). A ¹H NMR spectrum was recorded using a standard one-pulse experiment. Both the solid- and the liquid-state NMR spectra were collected with 32 k data points over a 10-ppm spectral width. The transmitter was set at the HDO frequency, which was also used as reference signal (4.79 ppm). The TopSpin 2.1 software package (Bruker) was used for data acquisition and processing of all spectra.

Size-exclusion high-pressure liquid chromatography (HPLC–SEC)

HPLC–SEC analysis was used to estimate the molecular size distribution of OAg populations. Samples were run, without pretreatment, on a TSK gel G3000 PWXL column (30 cm × 7.8 mm; particle size 7 µm; cod. 808021) with a TSK gel PWXL guard column (4.0 cm × 6.0 mm; particle size 12 µm; cod. 808033) (Tosoh Bioscience). The mobile phase was 0.1 M NaCl, 0.1 M NaH₂PO₄, 5% CH₃ CN, pH 7.2, at the flow rate of 0.5 mL/min (isocratic method for 30 min). Void and bed volume calibration was performed with λ-DNA (λ-DNA Molecular Weight Marker III 0.12–21.2 kb; Roche) and sodium azide (NaN₃; Merck), respectively. OAg peaks were detected by differential refractive index (dRI). UV detection at 214 and 260 nm was used for following impurity reduction during the purification process.

HPAEC–PAD

Rhamnose (Rha), galactose (Gal), glucose (Glc), and mannose (Man), each occurring once in the OAg chain repeating unit, and *N*-acetylglucosamine (GlcNAc), a sugar uniquely present in the core region, were estimated by HPAEC–PAD after acid hydrolysis of the OAg to release the monosaccharides [48]. Commercial monomer sugars were used for building the calibration curves. For Rha, Gal, Glc, and Man quantification, OAg samples, diluted to have each sugar monomer in the range 0.5–10 µg/mL, were hydrolyzed at 100 °C for 4 h in 2 M trifluoroacetic acid (TFA). This hydrolysis condition was optimal for release of all monomers without their degradation. For GlcNAc quantification, OAg samples, diluted to have a GlcNAc concentration in the range 0.5–10 µg/mL, were hydrolyzed at 100 °C for 6 h in 1 M TFA. After the hydrolysis, samples were chilled at 2–8 °C for about 30 min, dried by SpeedVac overnight, reconstituted in water, and filtered using 0.45-µm Acrodisc (PALL) filters before chromatographic analysis.

HPAEC–PAD was performed with a Dionex ICS3000 equipped with a CarboPac PA10 column (4 × 250 mm) coupled with a PA10 guard column (4 × 50 mm). Separation of the sugars was performed with a flow rate of 1 mL/min, eluting in a gradient from 10 to 18 mM NaOH over 20 min. After being washed for 20 min with 100 mM AcONa in 28 mM NaOH, the column was reequilibrated with 10 mM NaOH for 20 min. The effluent was monitored using an electrochemical detector in the pulse amperometric mode with a gold working electrode and an Ag/AgCl reference electrode. The Dionex standard quadruple-potential waveform for carbohydrates was used. The resulting chromatographic data were processed using Chromeleon software 6.8. Calibration curves were built for each sugar monomer (0.5–10 µg/mL). The standards were hydrolyzed and analyzed in the same way as samples. For GlcNAc, glucosamine was the species detected by HPAEC–PAD after hydrolysis.

KDO quantification by semicarbazide/HPLC–SEC method

OAg samples were analyzed by HPLC–SEC after derivatization with semicarbazide to quantify α-ketoacid present at the reducing end [48]. This reaction was performed as a slight modification of the semicarbazide assay for α-ketoacid determination [41]. OAg samples and KDO standards (100 µL of total volume in water), with a C=O concentration between 15.7 and 156.7 nmol/mL, were added to 100 µL of semicarbazide solution (100 mg semicarbazide hydrochloride + 90.5 mg of sodium acetate anhydrous in 10 mL of water). Sample blanks were prepared by adding 100 µL of sodium acetate (90.5 mg of sodium acetate anhydrous in 10 mL of water) to 100 µL of the OAg samples at the same concentration used for the analysis. All samples and standards were heated at 50 °C for 50 min and then analyzed by HPLC–SEC (80 µL injected), on a TSK gel G3000 PWXL column with guard column in 0.1 M NaCl, 0.1 M NaH₂PO₄, 5% CH₃CN, pH 7.2, mobile phase at the flow rate of 0.5 mL/min (isocratic method for 30 min). Detection was done at 252 nm. The peak area corresponding to OAg after derivatization with semicarbazide was

corrected by subtracting the area of the corresponding blank; the amount of KDO was calculated using the calibration curve built with the peak areas of derivatized KDO standard at 252 nm.

Results

Direct OAg extraction

Release of OAg from LPS takes advantage of the acid-labile glycosidic bond between the lipid A and the KDO at the end of the core region of LPS. Acetic acid hydrolysis of LPS is usually performed in 1% AcOH at 100 °C [2,3,5,8,23]; sodium acetate buffer at pH 4.5 has also been proven to efficiently cleave the lipid A–polysaccharide bond [42]. Here, AcOH was added directly to the bacterial culture hydrolyzing the OAg from the lipid A while still attached on the bacterial membrane. The O-antigen chain plus the core (Fig. 1) would be released in solution, while the lipid A would remain attached to the bacterial membranes and pelleted by centrifugation.

Acetic acid was added to the bacterial culture to give a 1–2% final concentration (v/v), with a resulting pH of 3.5–4.7, and the mixture was heated to 100 °C. For all strains, initial extraction tests were done at the 2-mL scale and the kinetics of hydrolysis was studied to optimize the duration of AcOH treatment at 100 °C.

Phenol sulfuric assay and LAL test

As shown in Table 2, the various strains released OAg in the culture medium over various hydrolysis times. For example *S. Paratyphi* A CVD1901 and *S. Enteritidis* 4314 reached a plateau of total sugar content in the supernatant after 2 h of hydrolysis, while for *S. Enteritidis* SA710, the hydrolysis was slower and the sugar content in the bacterial broth continued to increase up to 6 h. The amount of total sugar released in solution varied from serovar to serovar but was proportional to the culture OD (data not shown). In all cases, during hydrolysis endotoxin content in the supernatant was very low, confirming lipid A removal with bacteria by centrifugation. A small amount of LPS was presumably released during fermentation into the medium and accounted for the low sugar concentration and endotoxin levels detected at time 0.

Comparable results were obtained when the process was scaled up from 2 mL to 2 L and to 30 L. Table 2 shows the scale-up performed with *S. Paratyphi* A CVD1901, and similar results were obtained for all the other strains when scaled from 2 mL to 2 L (data not shown).

HR–MAS NMR

Proton HR–MAS NMR spectra were directly recorded on D23580 *S. Typhimurium* cells before performing AcOH treatment and on the bacteria recovered after complete hydrolysis. The spectrum recorded on the inactivated cells before hydrolysis showed the characteristic peaks of OAg saccharide, such as the anomeric protons at 5.0–5.6 ppm, the O-acetyl groups of Rha and Abe residues at approximately 2.0 ppm, and the methyl groups of 6-deoxy sugars, which resonate at about 1.2–1.3 ppm (Fig. 2A). These characteristic signals were absent after the extraction process (Fig. 2B), confirming that no residual OAg chains remained attached to the bacteria after the acid treatment. The ^1H NMR spectrum on purified O:4,5 in the liquid state was also collected as a positive control (Fig. 2C). The ^1H HR–MAS NMR spectrum collected on the cells (Fig. 2A) showed additional peaks not related to OAg saccharide (Fig. 2C). Proton signals of other molecules (peptido/glycan species) expressed on the surface of bacteria were revealed and sometimes they overlap with OAg peaks.

OAg purification

Development of the purification process with O:2 from *S. Paratyphi* A

CVD1901 (Table 1)—The purification process was developed with the aim of using steps suitable to scaling up and exploiting the specific physicochemical characteristics of both OAg and impurities released into the supernatant during the hydrolysis step.

- i. Recovery of high-molecular-mass OAg. CVD1901 produces OAg of varying molecular mass (MM) distribution. However, the profile of the “post hydrolysis supernatant” on HPLC–SEC (dRI) showed a well-defined relatively high MM population (Fig. 3) and this procedure was designed to specifically purify this species (indicated as HMM OAg). HPLC–SEC analysis (dRI) was used for quantifying the sugar content of this high MM population in all the purification steps, using purified HMM OAg as standard (concentration estimated by phenol sulfuric assay) for running the calibration curve in the range 25–500 µg/mL of carbohydrate. As shown in Table 3, high MM sugar recovery at each step in the process was high, with an overall recovery of the HMM OAg in the “post hydrolysis supernatant” of 80.4% at the 1-L scale and 82.7% at the 30-L scale after five purification steps.
- ii. Removal of impurities. The “post hydrolysis supernatant”, in addition to the HMM OAg, contained significant quantities of shorter saccharides (HMM OAg to total sugar content was determined to be 30–35% w/w), protein, and nucleic acid as well as low-molecular-mass impurities. These impurities, together with some of the lower MM sugars, proteins, and nucleic acids, were removed by the initial 30-kDa TFF step. Most of the residual proteins and a substantial proportion of the nucleic acids, together with part of the lower MM sugars, coprecipitated at pH 3 following concentration by TFF. Residual protein was largely removed by filtration through a Sartobind S filter. Residual nucleic acids and remaining non-HMM OAg sugars, with a higher negative charge density than high MM chains (Fig. 1), were removed with a calcium phosphate coprecipitation step.

Optimization of the calcium phosphate coprecipitation was required to obtain the results presented in Table 3. As shown in Table 4, a series of trial precipitations was done using “post-pH 3” material with a high percentage of protein and nucleic acid to HMM OAg (w/w). These tests were performed adding Na₂HPO₄ (32.7 mM before CaCl₂ addition) and increasing volumes of 5 M CaCl₂ up to 1.5 M (Table 4, samples 1A–4A). Using these conditions, protein content remained high and percentage of nucleic acid dropped to <1% only when CaCl₂ was 1 M. When 25% EtOH was included (before CaCl₂ addition) in the precipitation matrix (Table 4, samples 1B–4B), a reduction in both protein and nucleic acids was obtained even at 0.2 M CaCl₂. A precipitation performed without Na₂HPO₄ and with final CaCl₂ concentration of 1 M (Table 4, sample C) showed a reduction in protein and nucleic acids, but nucleic acid content was >10%. The addition of Na₂HPO₄ increased the pH to 5.5–6.0, which was then decreased to 4–4.5 by CaCl₂ addition (0.2 M final). The presence of phosphate was not needed to increase the pH, as the process also worked well adding NaH₂PO₄ at the same final concentration, maintaining the pH after phosphate addition at 3.3–3.5 (data not shown). Thus, for the precipitation of nucleic acids, the pH was not important, but the presence of the phosphate was. HMM OAg recoveries were similar for all the conditions tested.

As a result of these tests, 18 mM Na₂HPO₄, 24% EtOH, and 200 mM CaCl₂ were used in the purification process. These conditions were still not enough for reducing protein content to <1% working on post-pH 3 material, and an additional step of purification by Sartobind S was needed. A final TFF step was performed to remove salts and EtOH and to obtain the purified OAg in water.

- iii. 30-L scale. The purification process was scaled up to 30 L fermentation culture, keeping all the steps unchanged, but scaling up the surface of the TFF membranes and the Sartobind S filter. The purification process proved robust, giving results comparable in terms of recovery yields (approximately 150 mg of purified OAg per liter of fermentation broth) and purity (protein <1%, nucleic acid <1%, and endotoxin <0.1 EU/ μ g) to those obtained at the 1-L scale (Table 3).

Purification process applied to OAg from *S. Enteritidis* strains—The purification process developed for CVD1901 was applied to OAg from two different *S. Enteritidis* strains. Unlike the O:2 antigen from CVD1901, both D24359 and CMCC4313 produced two main OAg populations at relatively high MM (Fig. 4) that were copurified through the process, making the estimation of the recovery of the two OAg species by HPLC–SEC (dRI) complicated. For this reason, sugar recovery was calculated step by step with the phenol sulfuric assay and was not relative only to the OAg populations we were interested in purifying.

The reduction of nucleic acid and protein impurities during the process was followed and compared to the total sugar content (Table 5). High-quality material with low protein and nucleic acid impurities was obtained for both strains. Characterization of the final O:9 products (see OAg sample characterization) revealed very low contamination with either non-OAg sugars or OAg species at lower MM (HPAEC–PAD, ^1H NMR analysis, and semicarbazide assay). For O:9 purification from D24359, the percentage of residual protein was already very low (0.6%) in the “post-pH 3” sample; therefore, there was no need for the cation-exchange chromatography step through the Sartobind S filter. The overall total sugar recovery from D24359 was 67%; thus the recovery of the two O:9 populations at relatively high MM could not be lower than this.

In contrast, the recovery of total sugar from the “post hydrolysis supernatant” of CMCC4313 was only 17%. To investigate the cause of this low yield, OAg from CMCC4314 was purified using 5-kDa instead of 30-kDa MWCO membrane for the TFF-1 and TFF-2 steps. Total sugar recovery increased to 44%. The resulting purified material was separated on a Sephacryl S100 column (20 mg total sugar injected on a HiPrep Sephacryl S100 HR16/60 GE Healthcare; 50 mM NaH_2PO_4 , 150 mM NaCl, pH 7.2; 0.5 mL/min flow rate), confirming the presence of OAg populations at lower MM. Two new populations with an average of 2 and 8 repeating units per OAg chain were collected, in addition to the two species obtained using the TFF 30-kDa with averages of 32 and 77 units per chain (Fig. 5). TFF membranes of different MWCO may be used depending on the molecular size of the OAg to be purified.

Purification process applied to OAg from *S. Typhimurium* strains—For all the strains tested (D23580, NVGH1792, LT2, and 2192), the purification process worked well, in terms of recovery of sugar and purity (Table 6). As for the O:9 Enteritidis samples, the O:4,5 populations at relatively high MM, except for 2192, were heterogeneous (Fig. 4) and we were unable to estimate the sugar recovery of these species. Like with the D24359 strain, the recovery of total sugar was high for each step, and the characterization of the purified OAg (see OAg sample characterization) indicated low levels of non-OAg sugars and lower MM OAg. The overall recovery of high MM O:4,5 populations must also be higher than those calculated based on the total sugar.

As was obtained for the O:2 and O:9 purifications, these purified O:4,5 preparations had low levels of protein and nucleic acid impurities. The Sartobind S step was needed only for D23580; for all the other strains, protein content was sufficiently low in the “post-pH 3”

samples to have <1% protein impurities in the final products without performing the cationic-exchange chromatography step.

OAg sample characterization

All the purified OAg samples were characterized by ^1H NMR and HPAEC–PAD, confirming the presence of the expected sugars of the OAg chains (Table 7; Fig. 1). As commercial standards for Abe and Par are not available, while Tyv was unstable under the hydrolysis condition used for the quantification of the other monomers by HPAEC–PAD, the presence and amount of these sugars were determined by ^1H NMR.

Semicarbazide coupled with HPLC–SEC was used to verify the presence of the KDO sugar at the reducing end after OAg extraction and purification processes. For all the OAg purified from the various *Salmonella* strains, the amount of KDO was quantified and compared with GlcNAc, obtaining a molar ratio close to 1, as expected (Fig. 1; Table 7). The molar ratio Rha/KDO or Rha/GlcNAc indicates the average number of repeating units per OAg chain and, consequently, the average MM of the various OAg populations (Table 7). Fig. 4 shows HPLC–SEC profiles (dRI) for all the purified OAg samples, with OAg populations showing a molecular mass distribution consistent with the average number of repeating units obtained. The semicarbazide assay also confirmed the absence of OAg species at lower MM.

Discussion

A simplified and scalable methodology for the extraction and purification of the O-antigen core portion of LPS produced by various *Salmonella* strains has been developed. The suitability of the process at manufacturing scale will facilitate the robust generation of conjugate vaccines. Traditional methods for O-antigen extraction involve sedimentation of the bacteria, inactivation of the culture by formalin fixation, hot phenol extraction of the LPS [23], and treatment of the extracted LPS with acetic acid or anhydrous hydrazine [3] for LPS hydrolysis prior to O-antigen purification. Our method replaces this multistep process by performing acetic acid hydrolysis directly on bacteria fermentation cultures, resulting in the release of OAg into the culture supernatant. As shown by the kinetics of hydrolysis (Table 2) and by HR–MAS NMR (Fig. 2), at the end of the hydrolysis time OAg was completely released into the culture medium with negligible lipid A contamination (<0.1 EU/ μg for all the purified OAg) and with no saccharide chains still attached on the cell membranes. Since the direct acid hydrolysis method was very efficient with all the *Salmonella* serovar strains tested, we expect that the extraction method has broad applicability and can be potentially used to extract OAg from other Gram-negative bacteria. Similar results were obtained in our laboratory with OAg extracted from *Shigella sonnei* and OAg preparations are routinely obtained from additional *Salmonella* strains.

We also demonstrated that hydrolysis can take place directly in a sterilize-in-place industrial bioreactor, as verified with CVD1901 at the 30-L scale. This provides a much safer procedure compared with traditional methods, because it avoids: (a) manipulation of potentially pathogenic bacteria, with no need for cell isolation and/or inactivation prior to treatment, and (b) manipulation of large amounts of toxic phenol and dangerous LPS. OAg preparations at 30-L scale from *Salmonella* strains other than CVD1901 are routinely performed in our laboratories.

Acetic acid hydrolysis *per se* does not alter the O-acetylation levels of O-antigen, which can be critical for immunogenicity [3,43–46], and the whole extraction and purification process has been developed to retain the native properties of these potential vaccine antigens.

The purification process, built based on the structural characteristics of *Salmonella* O-antigen and generally applicable to neutral sugar chains, has proven to be robust and reproducible and has been applied with good results to various *S. enterica* serovars and strains. All steps are applicable for industrial scale-up and GMP manufacture. The process scaled to 30 L for *S. Paratyphi A* CVD 1901 gave results comparable to the purification performed on a smaller scale (Table 3). As shown for the purification of OAg from CMCC4314, substitution of TFF filters with a smaller cut-off allows purification of OAg populations at lower molecular mass. Working with capsulated strains, OAg populations could be easily separated from capsular polysaccharide, which would also be released into the growth medium after the hydrolysis step, using their different molecular masses.

Repeating-unit structure was verified for all the purified OAg (Table 7) and the presence of GlcNAc and KDO in a molar ratio close to 1 confirmed that the process had cleaved the lipid A, leaving one KDO sugar per O-antigen chain. The KDO at the terminus is available for conjugation to carrier proteins. Considering that the O-antigen chain remains unaltered by the mild extraction and purification process, conjugates made using this method would be particularly desirable as vaccine candidates.

In summary, an OAg extraction and purification process that is quicker, safer, more environmentally friendly, and less expensive than traditionally used methods has been developed. The demonstrated effectiveness and suitability for large-scale production make the improved process relevant for those working in the fields of OAg structure, immunogenicity, and vaccinology.

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References

- [1]. Carlin NI, Svenson SB, Lindberg AA. Role of monoclonal O-antigen antibody epitope specificity and isotype in protection against experimental mouse typhoid. *Microb. Pathog.* 1987; 2:171–183. [PubMed: 2467161]
- [2]. Watson DC, Robbins JB, Szu SC. Protection of mice against *Salmonella typhimurium* with an O-specific polysaccharide–protein conjugate vaccine. *Infect. Immun.* 1992; 60:4679–4686. [PubMed: 1383154]
- [3]. Konadu E, Shiloach J, Bryla DA, Robbins JB, Szu SC. Synthesis, characterization, and immunological properties in mice of conjugates composed of detoxified lipopolysaccharide of *Salmonella paratyphi A* bound to tetanus toxoid with emphasis on the role of O acetyls. *Infect. Immun.* 1996; 64:2709–2715. [PubMed: 8698499]
- [4]. Simon R, Tennant SM, Wang JY, Schmidlein PJ, Lees A, Ernst RK, Pasetti MF, Galen JE, Levine MM. *Salmonella enterica* serovar enteritidis core O polysaccharide conjugated to H:g, m flagellin as a candidate vaccine for protection against invasive infection with *S. enteritidis*. *Infect. Immun.* 2011; 79:4240–4249. [PubMed: 21807909]
- [5]. Chu CY, Liu BK, Watson D, Szu SS, Bryla D, Shiloach J, Schneerson R, Robbins JB. Preparation, characterization, and immunogenicity of conjugates composed of the O-specific polysaccharide of *Shigella dysenteriae* type 1 (Shiga's bacillus) bound to tetanus toxoid. *Infect. Immun.* 1991; 59:4450–4458. [PubMed: 1937803]
- [6]. Taylor DN, Trofa AC, Sadoff J, Chu C, Bryla D, Shiloach J, Cohen D, Ashkenazi S, Lerman Y, Egan W, et al. Synthesis, characterization, and clinical evaluation of conjugate vaccines composed of the O-specific polysaccharides of *Shigella dysenteriae* type 1, *Shigella flexneri* type

- 2a, and *Shigella sonnei* (*Plesiomonas shigelloides*) bound to bacterial toxoids. *Infect. Immun.* 1993; 61:3678–3687. [PubMed: 8359890]
- [7]. Passwell JH, Ashkenazi S, Harlev E, Miron D, Ramon R, Farzam N, Lerner-Geva L, Levi Y, Chu C, Shiloach J, Robbins JB, Schneerson R. Israel Shigella study group, safety and immunogenicity of *Shigella sonnei*-CRM9 and *Shigella flexneri* type 2a-rEPAsucc conjugate vaccines in one- to four-year-old children. *Pediatr. Infect. Dis. J.* 2003; 22:701–706. [PubMed: 12913770]
- [8]. Konadu E, Robbins JB, Shiloach J, Bryla DA, Szu SC. Preparation, characterization, and immunological properties in mice of *Escherichia coli* O157 O-specific polysaccharide–protein conjugate vaccines. *Infect. Immun.* 1994; 62:5048–5054. [PubMed: 7927787]
- [9]. Wang X, Zhang C, Shi F, Hu X. Purification and characterization of lipopolysaccharides. *Subcell. Biochem.* 2010; 53:27–51. [PubMed: 20593261]
- [10]. Apicella MA. Isolation and characterization of lipopolysaccharides. *Methods Mol. Biol.* 2008; 431:3–13. [PubMed: 18287743]
- [11]. Staub, AM. Bacterial lipido-proteinopolysaccharides (“O” somatic antigens): extraction with trichloroacetic acid. In: Whistler, RLWM., editor. *Methods in Carbohydrate Chemistry*. Academic Press; New York: 1965. p. 92
- [12]. Ribí E, Haskins WT, Landy M, Milner KC. Preparation and host-reactive properties of endotoxin with low content of nitrogen and lipid. *J. Exp. Med.* 1961; 114:647–663. [PubMed: 14491836]
- [13]. Leive L. Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetate. *J. Biol. Chem.* 1968; 243:2373–2380. [PubMed: 4967584]
- [14]. Morrison DC, Leive L. Fractions of lipopolysaccharide from *Escherichia coli* O111:B4 prepared by two extraction procedures. *J. Biol. Chem.* 1975; 250:2911–2919. [PubMed: 804483]
- [15]. Uchida K, Mizushima S. A simple method for isolation of lipopolysaccharides from *Pseudomonas aeruginosa* and some other bacterial strains. *Agric. Biol. Chem.* 1987; 51:3107–3114.
- [16]. Sonesson A, Jantzen E, Bryn K, Larsson L, Eng J. Chemical composition of a lipopolysaccharide from *Legionella pneumophila*. *Arch. Microbiol.* 1989; 153:72–78. [PubMed: 2610584]
- [17]. Goebel WF, Binkley F, Perlman E. Studies on the flexner group of dysentery bacilli. I. The specific antigens of *Shigella paradyserteriae* (Flexner). *J. Exp. Med.* 1945; 81:315–330. [PubMed: 19871460]
- [18]. Eidhin DN, Mouton C. A rapid method for preparation of rough and smooth lipopolysaccharide from *Bacteroides*, *Porphyromonas* and *Prevotella*. *FEMS Microbiol. Lett.* 1993; 110:133–138. [PubMed: 8349089]
- [19]. Mirzae A, Hedayati E, Reza H, Ashtiani A, Rahbar M, Rastegar H. A simple method for non phenolic extraction of lipopolysaccharide from *Salmonella typhimurium* and *Salmonella enteritidis* with high purity and pyrogenicity in rat. *Sci. Res. Essays.* 2011; 6:1101–1105.
- [20]. Galanos C, Luderitz O, Westphal O. A new method for the extraction of R lipopolysaccharides. *Eur. J. Biochem.* 1969; 9:245–249. [PubMed: 5804498]
- [21]. Nurminen M, Vaara M. Methanol extracts LPS from deep rough bacteria. *Biochem. Biophys. Res. Commun.* 1996; 219:441–444. [PubMed: 8605006]
- [22]. Darveau RP, Hancock RE. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. *J. Bacteriol.* 1983; 155:831–838. [PubMed: 6409884]
- [23]. Westphal, O.; Jann, K. Bacterial lipopolysaccharides: extraction with phenolwater and further applications of the procedure. In: Whistler, RL., editor. *Methods in Carbohydrate Chemistry*. Academic Press; New York: 1965. p. 83-91.
- [24]. Johnson KG, Perry MB. Improved techniques for the preparation of bacterial lipopolysaccharides. *Can. J. Microbiol.* 1976; 22:29–34. [PubMed: 814982]
- [25]. Simin R, Tennant SM, Wang JY, Schmidlein PJ, Lees A, Ernst RK, Pasetti MF, Galen JE, Levine MM. Extraction, purification and characterization of lipopolysaccharide from *Escherichia coli* and *Salmonella typhi*. *Avicenna J. Med. Biotechnol.* 2011; 3:3–9. [PubMed: 23407691]
- [26]. Nalbantsoy A, Karaboz I, Ivanova R, Deliloglu-Gurhan I. Isolation and purification of O and H antigens from *Salmonella enteritidis* as diagnostic tool. *Ann. Microbiol.* 2010; 60:565–571.

- [27]. Kubler-Kielb J, Vinogradov E, Ben-Menachem G, Pozsgay V, Robbins JB, Schneerson R. Saccharide/protein conjugate vaccines for *Bordetella* species: preparation of saccharide, development of new conjugation procedures, and physico-chemical and immunological characterization of the conjugates. *Vaccine*. 2008; 26:3587–3593. [PubMed: 18539367]
- [28]. Gupta RK, Szu SC, Finkelstein RA, Robbins JB. Synthesis, characterization, and some immunological properties of conjugates composed of the detoxified lipopolysaccharide of *Vibrio cholerae* O1 serotype Inaba bound to cholera toxin. *Infect. Immun.* 1992; 60:3201–3208. [PubMed: 1639490]
- [29]. Kondo S, Hisatsune K. Rapid preparation of samples for compositional sugar analysis of the “degraded polysaccharide” fraction of lipopolysaccharides from *Vibrio cholerae*. *Microbiol. Immunol.* 1988; 32:907–915. [PubMed: 3210996]
- [30]. Hisatsune K, Iguchi T, Kondo S. A rapid method for sugar analysis of lipopolysaccharides of gram-negative bacteria. *System. Appl. Microbiol.* 1990; 13:320–326.
- [31]. Rahman MM, Guard-Petter J, Carlson RW. A virulent isolate of *Salmonella enteritidis* produces a *Salmonella* typhi-like lipopolysaccharide. *J. Bacteriol.* 1997; 179:2126–2131. [PubMed: 9079895]
- [32]. Hellerqvist CG, Lindberg B, Svensson S, Holme T, Lindberg AA. Structural studies on the O-specific side-chains of the cell-wall lipopolysaccharide from *Salmonella typhimurium* LT2. *Carbohydr. Res.* 1969; 9:237–241.
- [33]. Gat O, Galen JE, Tennant S, Simon R, Blackwelder WC, Silverman DJ, Pasetti MF, Levine MM. Cell-associated flagella enhance the protection conferred by mucosally-administered attenuated *Salmonella paratyphi* A vaccines. *PLoS Negl. Trop. Dis.* 2011; 5:e1373. [PubMed: 22069504]
- [34]. MacLennan CA, Gondwe EN, Msefula CL, Kingsley RA, Thomson NR, White SA, Goodall M, Pickard DJ, Graham SM, Dougan G, Hart CA, Molyneux ME, Drayson MT. The neglected role of antibody in protection against bacteremia caused by nontyphoidal strains of *Salmonella* in African children. *J. Clin. Invest.* 2008; 118:1553–1562. [PubMed: 18357343]
- [35]. Kingsley RA, Msefula CL, Thomson NR, Kariuki S, Holt KE, Gordon MA, Harris D, Clarke L, Whitehead S, Sangal V, Marsh K, Achtman M, Molyneux ME, Cormican M, Parkhill J, MacLennan CA, Heyderman RS, Dougan G. Epidemic multiple drug resistant *Salmonella typhimurium* causing invasive disease in sub-Saharan Africa have a distinct genotype. *Genome Res.* 2009; 19:2279–2287. [PubMed: 19901036]
- [36]. Beltran P, Musser JM, Helmuth R, Farmer JJ III, Frerichs WM, Wachsmuth IK, Ferris K, McWhorter AC, Wells JG, Cravioto A, et al. Toward a population genetic analysis of *Salmonella*: genetic diversity and relationships among strains of serotypes *S. choleraesuis*, *S. derby*, *S. dublin*, *S. enteritidis*, *S. heidelberg*, *S. infantis*, *S. newport*, and *S. typhimurium*. *Proc. Natl. Acad. Sci. USA.* 1988; 85:7753–7757. [PubMed: 3051004]
- [37]. Beltran P, Plock SA, Smith NH, Whittam TS, Old DC, Selander RK. Reference collection of strains of the *Salmonella typhimurium* complex from natural populations. *J. Gen. Microbiol.* 1991; 137:601–606. [PubMed: 2033380]
- [38]. Lilleengen K. Typing of *Salmonella dublin* and *Salmonella enteritidis* by means of bacteriophage. *Acta Pathol. Microbiol. Scand.* 1950; 27:625–640. [PubMed: 14770857]
- [39]. Rondini S, Micoli F, Lanzilao L, Hale C, Saul AJ, Martin LB. Evaluation of the immunogenicity and biological activity of the *Citrobacter freundii* Vi-CRM197 conjugate as a vaccine for *Salmonella enterica* serovar typhi. *Clin. Vaccine Immunol.* 2011; 18:460–468. [PubMed: 21248155]
- [40]. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 1956; 28:350.
- [41]. Macgee J, Doudoroff M. A new phosphorylated intermediate in glucose oxidation. *J. Biol. Chem.* 1954; 210:617–626. [PubMed: 13211598]
- [42]. Rosner MR, Tang J, Barzilay I, Khorana HG. Structure of the lipopolysaccharide from an *Escherichia coli* heptose-less mutant. I. Chemical degradations and identification of products. *J. Biol. Chem.* 1979; 254:5906–5917. [PubMed: 221486]

- [43]. Berry DS, Lynn F, Lee CH, Frasch CE, Bash MC. Effect of O acetylation of *Neisseria meningitidis* serogroup A capsular polysaccharide on development of functional immune responses. *Infect. Immun.* 2002; 70:3707–3713. [PubMed: 12065513]
- [44]. Orskov F, Orskov I, Sutton A, Schneerson R, Lin W, Egan W, Hoff GE, Robbins JB. Form variation in *Escherichia coli* K1: determined by O-acetylation of the capsular polysaccharide. *J. Exp. Med.* 1979; 149:669–685. [PubMed: 372481]
- [45]. Avery OT, Goebel WF. Chemoimmunological studies on the soluble specific substance of *Pneumococcus*. I. The isolation and properties of the acetyl polysaccharide of pneumococcus type I. *J. Exp. Med.* 1933; 58:731–755. [PubMed: 19870226]
- [46]. Szu SC, Li XR, Stone AL, Robbins JB. Relation between structure and immunologic properties of the Vi capsular polysaccharide. *Infect. Immun.* 1991; 59:4555–4561. [PubMed: 1937814]
- [47]. Jansson PE, Lindberg AA, Lindberg B, Wollin R. Structural studies on the hexose region of the core in lipopolysaccharides from Enterobacteriaceae. *Eur. J. Biochem.* 1981; 115:571–577. [PubMed: 7016539]
- [48]. Micoli F, Rondini S, Gavini M, Lanzilao L, Medaglini D. O:2-CRM197 Congugates against *Salmonella* Paratyphi A. *PLoS One.* 2012; 7:e47039. [PubMed: 23144798]

Fig. 2. HR-MAS NMR analysis of (A) *S. Typhimurium* D23580 cells and (B) D23580 cells after treatment with acetic acid in comparison with (C) ¹H NMR of D23580 OAg in the liquid state.

Fig. 3. HPLC-SEC profile (dRI) of the “post hydrolysis supernatant” from *S. Paratyphi A* CVD1901.

The arrow indicates the OAg population at relatively high MM that was purified through the process. Samples were run on a TosoHaas TSK gel 3000 PWXL column; eluent, 0.1 M NaH_2PO_4 , 0.1 M NaCl, 5% CH_3CN , pH 7.2; flow rate, 0.5 mL/min; column void volume, 10.85 min; total volume, 23.54 min.

Fig. 4. HPLC-SEC profiles (dRI) of purified OAg from (A) *S. Paratyphi* A CVD1901, (B) *S. Enteritidis* D24359, (C) *S. Enteritidis* CMCC4314, (D) *S. Typhimurium* 2192, and (E) *S. Typhimurium* D23580, NVGH1792, and LT2.

Samples were run on a TosoHaas TSK gel 3000 PWXL column; eluent, 0.1 M NaH_2PO_4 , 0.1 M NaCl, 5% CH_3CN , pH 7.2; flow rate, 0.5 mL/min; column void volume, 10.85 min; total volume, 23.54 min.

Fig. 5. HPLC-SEC profiles (dRI) of the populations at varying MM collected by running purified OAg from *S. Enteritidis* CMCC4314 on Sephacryl S100. Solid line, population with 77 OAg chain repeats according to HPAEC-PAD analysis; dashed line, population with 32 OAg chain repeats; dotted line, population with 8 OAg chain repeats; dash-dot line, population with 2 OAg chain repeats. Samples were run on a TosoHaas TSK gel 3000 PWXL column; eluent, 0.1 M NaH_2PO_4 , 0.1 M NaCl, 5% CH_3CN , pH 7.2; flow rate, 0.5 mL/min; column void volume, 10.85 min; total volume, 23.54 min.

Table 1
Flow chart for OAg purification process.

Step	Notes
Hydrolysis (1–2% AcOH, pH 3.5–4.7; 100 °C, $t < 6$ h) and neutralization of the fermentation broth; harvest by centrifugation	OAg hydrolysis directly from intact bacteria avoiding the step of LPS extraction
TFF 30 kDa	Concentration of 5- to 10-fold; diafiltration against 20 diavolumes of 1 M NaCl and 10 diavolumes of water; removal of lower molecular mass impurities
Protein–nucleic acid coprecipitation at pH 3; clarification by centrifugation	Reduction of protein and nucleic acid impurities
Cation-exchange chromatography (Sartobind S) ^a	Further reduction of protein impurities
Precipitation adding Na ₂ HPO ₄ (18 mM final), EtOH (24% final), and 5 M CaCl ₂ (200 mM final); clarification by centrifugation	Further reduction of nucleic acid impurities
TFF 30 kDa	Concentration up to 10-fold and diafiltration against 10 diavolumes of water
Final 0.22-μm filtration	

^aSartobind S step was included only if the residual protein content after the precipitation at pH 3 was >3% (w/w respect to total sugar content).

Table 2
Kinetics of OAg extraction for various *Salmonella* strains at various times of hydrolysis.

OAg serovar:	O:2 from <i>S. Paratyphi A</i>			O:4,5 from <i>S. Typhimurium</i>			O:9 from <i>S. Enteritidis</i>						
Strain:	CVD 1901 ^a	2 mL	2 L	30 L	2 mL	2 mL	2 mL	2 mL	2 mL	2 mL	2 mL	SA710	SA386
Extraction scale:	2 mL	2 mL	2 L	30 L	2 mL	2 mL	2 mL	2 mL	2 mL	2 mL	2 mL	2 mL	2 mL
Total sugar at time 0, µg/mL (EU/µg endotoxin)	nd	nd	nd	nd	317 (5832)	75 (7 × 10 ⁴)	nd	451	14 (190)	76 (1377)			
Total sugar after 2 h hydrolysis, µg/mL (EU/µg endotoxin)	534 (<0.5)	563 (<0.5)	530 (<0.5)	530 (<0.5)	1355 (<0.1)	954 (<0.1)	644 (3.9)	431	1277	345	618 (<0.1)		
Total sugar after 4 h hydrolysis, µg/mL (EU/µg endotoxin)	532	629	582	582	1440 (<0.1)	1023 (<0.1)	734	413	1169	572	756 (<0.1)		
Total sugar after 6 h hydrolysis, µg/mL (EU/µg endotoxin)	593 (<0.1)	621 (<0.1)	630 (<0.1)	630 (<0.1)	1624 (<0.1)	1183 (<0.1)	753 (<0.1)	455 (<0.1)	1383 (<0.1)	832 (<0.1)	862 (<0.1)		

Total sugar and endotoxin contents were quantified in post hydrolysis supernatants after desalting against water on columns prepacked with Sephadex G-25 Superfine. The values reported are corrected for the dilution factor due to the desalting. nd, not determined.

^a Hydrolysis was performed at different scales on three different fermentations.

Table 3

Efficiency of O:2-antigen purification process.

Sample	Purification 1-L scale				Purification 30-L scale			
	Recovery % total OAg	Recovery % HMM OAg	% Protein ^a	% Nucleic acid ^a	Recovery % total OAg	Recovery % HMM OAg	% Protein ^a	% Nucleic acid ^a
Post hydrolysis supernatant	–	–	478	540	–	–	315	415
TFF-1 retentate	70	100	256	283	64	94	665	253
Post-pH 3	73	100	14	92	89	100	9.2	165
Post-Sartobind S	92	94	2.1	91	95	100	2.0	158
Post-EtOH/CaCl ₂	63	94	0.5	0.2	nd	nd	nd	nd
TFF-2 retentate and post-0.22 μm	100	91	0.4	<0.1	68	88	0.3	0.2

^a Calculated with respect to the HMM OAg content (w/w). HMM OAg content was quantified by HPLC-SEC, total sugar content by phenol sulfuric assay, protein content by micro-BCA, and nucleic acid by A260. Sugar recovery was calculated with respect to the previous step.

Table 4
Precipitation tests on post-pH 3 sample to reduce nucleic acid impurities.

Sample	Na ₂ HPO ₄ , mM	% EtOH	CaCl ₂ , M	% Protein ^a	% Nucleic acid ^a
Pretreatment	–	–	–	9.2	147.1
1A	32.7	None	0.2	9.6	12.4
2A	32.7	None	0.5	10.9	6.0
3A	32.7	None	1.0	10.1	0.4
4A	32.7	None	1.5	9.9	0.5
1B	32.7	25	0.2	3.0	0.2
2B	32.7	25	0.5	5.4	0.2
3B	32.7	25	1.0	5.4	0.1
4B	32.7	25	1.5	5.6	0.4
C	None	25	1.0	3.1	11.8

The concentration of Na₂HPO₄ was 32.7 mM before CaCl₂ (A) or EtOH (B) addition; percentage of EtOH was 25% before CaCl₂ addition (B and C).

^a Calculated with respect to the HMM OAg content (w/w) in the supernatant recovered after precipitation of impurities.

Table 5
Efficiency of O:9-antigen purification process.

Sample	% Yield sugar		% Protein ^a		% Nucleic acid ^a	
	CMCC4314	D24359	CMCC4314	D24359	CMCC4314	D24359
Post hydrolysis supernatant			103.7	28.9	103.7	41.2
TFF-1 retentate	nd	100	nd	3.8	nd	13.8
Post-pH 3	41.1	83.6	7.1	0.6	7.1	10.9
Post-Sartobind S	96.6	_ ^b	0.9	_ ^b	0.9	_ ^b
Post-EtOH/CaCl ₂	57.5	93.3	0.7	0.5	0.7	0.3
TFF-2 retentate and post-0.22 μm	72.8	86.2	0.7	0.4	0.7	0.18

^aCalculated with respect to the total sugar content (w/w). Total sugar content was quantified by phenol sulfuric assay, protein content by micro-BCA, and nucleic acid by A₂₆₀. Sugar recovery was calculated with respect to the previous step. In CMCC4314 the “post-pH 3” recovery was calculated with respect to the “post hydrolysis supernatant”.

^bThe Sartobind S step was not required for purification from this strain.

Table 6

Efficiency of O:4,5-antigen purification process.

Sample	% Sugar recovery				% Protein ^a				% Nucleic acid ^a			
	2192	D23580	LT2	NVGH1792	2192	D23580	LT2	NVGH1792	2192	D23580	LT2	NVGH1792
Post hydrolysis supernatant					27.1	76.1	47.1	34.4	52.8	101.1	61.8	39.5
TFF-1 retentate	96.6	57.2	82.0	79.9	24.4	20.2	16.9	11.0	61.8	68.5	76.8	19.6
Post-pH 3	90.2	100	95.5	94.9	0.7	6.6	2.6	3.1	41.9	42.8	62.9	11.7
Post-Sartobind S	– ^b	91.9	– ^b	– ^b	– ^b	0.7	– ^b	– ^b	– ^b	41.4	– ^b	– ^b
Post-EtOH/CaCl ₂	70.2	90.6	67.3	76.5	1.0	0.4	1.9	2.9	0.2	1.0	0.4	0.3
TFF-2 retentate and post-0.22 µm	77.2	91.7	94.4	94.2	0.9	0.4	0.4	0.6	0.7	0.15	0.22	0.13

^a Calculated with respect to the total sugar content (w/w). Total sugar content quantified by phenol sulfuric assay, protein content by micro-BCA, and nucleic acid by A260. Sugar recovery was calculated with respect to the previous step.

^b Sartobind S step was not required for purification from this strain.

Table 7
Purified OAg sugar composition analysis.

OAg	Strain	Molar ratio					
		Man/Rha	Gal/Rha	Glc/Rha	Tyv (Par or Abe)/Rha	Rha/GlcNAc	KDO/GlcNAc
O:2	CVD1901	1.07	1.05	0.74	1.06	67	0.71
O:9	CMCC4314 ^a	0.92	1.0	0.08	0.88	76.7 (HMM); 32.2 (MMM)	nd
	D24359 ^a	0.98	1.04	0.09	0.97	40.5	1.08
O:4,5	2192	0.99	1.06	0.24	1.03	28	0.98
	D23580 ^a	1.05	1.08	0.41	1.04	71.0 (HMM); 25.5 (MMM)	1.09
	LT2 ^a	1.03	1.27	0.11	1.00	93.6 (HMM); 32.3 (MMM)	1.06
	NVGH1792 ^a	1.02	1.22	0.08	1.07	87.0 (HMM); 34.7 (MMM)	0.90

Rha, Man, Gal, Glc, and GlcNAc were quantified by HPAEC–PAD, KDO was quantified by semicarbazide/HPLC–SEC analysis, and Tyv (Par or Abe) ratios were determined by ¹H NMR.

^aThis strain produced two main OAg populations that were copurified. The ratio Rha/GlcNAc was calculated for the two species separated by size-exclusion chromatography and indicated as high MM (HMM) and medium MM (MMM) populations. HMM and MMM OAg were identical in terms of composition of the repeating unit. For D24359 the HMM population was negligible in terms of moles and the Rha/GlcNAc ratio indicates the length of the species at MMM only. For all the samples the KDO/GlcNAc ratio was calculated based on the total amount of monomers in the sample, without separating HMM and MMM species.